

Appendix A (Application No.: 10/682584)

Teachings In the Originally Filed Specification Showing That the Presently Claimed Effects and Therapeutic Results Are Dose-Dependent

The originally filed specification presents results of actual treatments of human tumors *in vivo* to state explicitly that the presently claimed subject matter is dose-dependent.

See e.g. page 12, lines 5-10 stating that “... *microscopic appearances of the tumor areas subjected to surgical excisions ... when the BCC’s lost most of their pre-treatment areas but still possessed few regions that, although markedly decreased in height, had not yet completely disappeared and therefore had residual tumor cells for microscopic analyses*”.

Disappearance of most of the pre-treatment areas of a tumor and decrease in height from skin surface are stated to be caused by the treatment and it is also stated that complete disappearance of a tumor that occurs with continuation of medicament administration was not allowed in the examples shown in Figures 2A to 2F so as to have residual tumor cells for microscopic analyses. Figures 1A to 1D also show causations of the decrease of size as well as visual disappearance of several tumor areas on fifth and sixth days of cyclopamine applications.

A person skilled in the art knows that a topically applied hydrophobic compound (cyclopamine) generates a concentration gradient where the highest concentration exists near epidermis and that the concentration decreases towards deeper dermis. From the exemplification in Figure 2A, the skilled artisan sees that the tumor nodule adjacent the epidermis has disappeared to leave behind a large cystic structure with no detectable tumor cells therein and that the epidermis above it (covering it) has normal morphology. In this particular example, the deep dermis receiving a relatively lower dose of cyclopamine is seen to have small tumor nodules wherein tumor cell apoptosis has started to cause formation of cystic spaces but there are still persisting tumor cells (Figure 2A). Thus, the skilled artisan sees that induction of apoptosis of tumor cells is dose-dependent.

Topical administration of cyclopamine onto only a half of a skin tumor followed by the excision of entire tumor for analyses provides a further objective means of assessing effects of different doses on tumor cells in the same patient. This is because the topically applied cyclopamine diffuses not only towards deeper dermis but also horizontally to the adjacent half of

tumor. Page 15, line 26 to page 16, line 18 describes results of such an investigation. It is seen from Figures 5A and 5B that *“While rapid regression of the tumor is evident in the cyclopamine-applied lower half, the region of tumor furthest away from the directly applied half is seen to be relatively unaltered”* (page 16, lines 4-7). As indicated, towards the uppermost part of said tumor, the tumor cells will be exposed to relatively much lower concentrations of cyclopamine. Thus, the skilled artisan sees that causation of tumor regression or shrinkage is dose-dependent. Similarly, the treatment results described on page 17, line 16 to page 19, line 4 show that causations of the presently described and claimed effects and therapeutic results are dose-dependent. The clinical photographs shown in Figures 7A and 7B show that the tumor nodules onto which cyclopamine was applied directly had largely regressed or disappeared while a nodule onto which cyclopamine was not applied directly was not visibly decreased in size.

Page 16, lines 2-18 and Figures 5A to 5F describe that induction of tumor cell apoptosis and the consequent decrease of tumor size are clearly dose-dependent. It is further seen from Figures 5E and 5F that a dose of cyclopamine causing complete disappearance of the Ki-67 labeling of tumor cells left behind still persisting tumor cells, indicating that elimination of tumor cells by induction of apoptosis requires a dose greater than a dose effective to inhibit proliferation of the tumor cells. Ki-67 is a well known marker of proliferating cells. Other, quantitative analyses of the proliferating and apoptosing tumor cells in various regions of the tumor shown in Figure 5B have been presented with the 1.10.2007 dated amendment and also show that causation of apoptosis of tumor cells in a patient requires a dose of cyclopamine surpassing a dose effective to inhibit proliferation of tumor cells (see paragraphs 26-28 and Figures 1A, 1B, 2A, 2B in the Declaration filed therewith).

Page 6, lines 21-30 and page 15, lines 4-8 refer to Figures 4A, 4B, 4C and 4D which show that normal skin tissue on and around the cyclopamine-treated BCC's exposed to the same cyclopamine doses as BCC's showed preservation of the normal Ber-Ep4 labeling of epidermal basal layer cells and of hair follicle cells. The skilled artisan knows that the epidermis basal layer in the epidermal invaginations or ridges has continuity with the hair follicle outer root sheath basal layer and contains undifferentiated cells having stem cell and progenitor cell features. Since the monoclonal antibody Ber-Ep4 was known to detect an antigen (EpCAM) expressed by most cancers and by the normal epithelial cells displaying stem cell and progenitor cell features in skin

and in other tissues (see e.g. Jimenez FJ et al, *Modern Pathology* 1995;8:854-858; deBoer CJ et al, *Journal of Pathology* 1999;188:201-206; Kubuschok B et al, *Journal of Clinical Oncology* 1999;17:19-24), skilled artisan knows that the Ber-Ep4 labeled normal cells in the epidermal ridges and hair follicles exposed to cyclopamine (Figures 4A to 4F) must represent normal cells having stem cell and progenitor cell features. Because these normal cells were exposed to the same or even greater doses of cyclopamine as the adjacent apoptosing tumor cells showing differentiation of all residual cells (see page 5, lines 21-30 and Table I), the skilled artisan immediately realizes that what is described is unprecedented and highly advantageous in treatment of cancer. The specification states that sparing of said undifferentiated normal cells following a dose eliminating tumor cells provides information about doses in other possible modes of administration, e.g. systemic administration, as well (page 11, lines 22-27). The specification also reminds that optimal dosing and application schedules are affected by factors well known in art and can be determined by known optimization methods (page 11, lines 10-15). Finally, it is indicated that “*Causation of highly efficient differentiation and apoptosis of the tumor cells in vivo ... at doses that preserve the undifferentiated tissue cells ... support the use of cyclopamine not only on BCC’s but also on those internal tumors that utilize the hedgehog/smoothened pathway ... for prevention of apoptosis and/or differentiation*” (page 15, lines 19-24).

Figure 7F showing a gradient pattern of Ber-Ep4 labeling in tissue shows most dramatically that induction of tumor cell differentiation *in vivo* is dose-dependent (see page 18, line 21 to page 19, line 4). Since the tissue shown in Figure 7F was excised six days after an initial three days of cyclopamine administration to the nearby tumor nodules (page 17, lines 16-28), it is further clear and instructive that the tumor cells induced to differentiate had not reverted during the six days of follow up without cyclopamine administration. In addition, as described (page 18, lines 4-10), the residual tumor cells particularly near the edges of cystic spaces formed by the apoptotic elimination of tumor cells had unusually high frequencies of cells showing spinous differentiation. Thus, the tumor cells receiving a dose of cyclopamine just below a dose effective to induce apoptosis were committed to differentiation as shown by their differentiation all the way to the terminally differentiated spinous cells during the six days of non-treated follow up. These results further show that induction of apoptosis of tumor cells requires a dose that is even greater than a dose inducing differentiation. The other evidence is the fact that when skin tissues

corresponding to the pre-treatment positions of tumors were excised after regression or disappearance of most tumor areas but before complete tumor disappearance so as to have residual tumor cells for microscopic analyses (page 12, lines 5-10), all residual tumor cells were differentiated by the criterion of Ber-Ep4 labeling but there were still tumor cells not yet apoptosed (see e.g. Table I). Since the loss of Ber-Ep4 labeling reflects a stage of differentiation just after the multipotential progenitor cell stage (Jimenez et al, *ibid*; deBoer et al, *ibid*; Kubuschok et al, *ibid*), it is clear that when a sufficient dose of cyclopamine is administered, tumor cells are induced to differentiate to an early stage revealed by Ber-Ep4 but the differentiating tumor cells are then eliminated by apoptosis before they have an opportunity to proceed all the way to the terminally differentiated cells. When the cyclopamine administration is discontinued before the tumor cells induced to differentiate undergo apoptosis, on the other hand, such tumor cells can proceed all the way to the terminally differentiated cells (page 17, line 16 to page 18, line 10; Figures 7C, 7D).

As summarized on page 19, lines 2-5, inductions of differentiation and apoptosis of tumor cells is not possible with suboptimal concentrations or doses of cyclopamine. The skilled artisan also notes that duration and amount of cyclopamine administered to a tumor-bearing patient effective to eliminate tumor cells by apoptosis will cause apoptosis and/or differentiation of much smaller proportion or no normal cells and this facilitates treatment of internal tumors (see e.g. page 15, lines 19-24; page 19, line 21 to page 20, line 8).